Prevalence, phenotypic and Genotypic Characterization of Vancomycin Resistant Enterococci (VRE) isolated from Children with Infective Endocarditis in Mansoura University Children's Hospital (MUCH), Mansoura, Egypt.

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ABSTRACT

Introduction*: Despite improvements in medical and surgical therapies, infective endocarditis among children, is still associated with poor prognosis and remains as a strong therapeutic challenge. Many factors affect the outcome of this serious disease, including virulence of the causative microorganism, characteristics of the patients, presence of underlying disease, delays in diagnosis and treatment, surgical indications, and timing of surgery. Vancomycin-resistant enterococci (VRE) pose an emerging and truly, a serious problem in hospitals worldwide.* ***Aim of the work****: This study was undertaken to determine the occurrence, species prevalence, antibacterial resistance, phenotypic and genetic characteristics of VRE isolated from children with infective endocarditis in Mansoura, Egypt.* ***Material and Methods:*** *Fifty isolates of enterococcal species were obtained from 358 blood samples collected from 350 patients of suspected infective endocarditis among children admitted to Mansoura University Children's Hospital (MUCH) cardiology unit and pediatric intensive care unit (PICU) over 6 years of study. The antibiotic susceptibility of isolates and minimum inhibitory concentration (MIC) tests for vancomycin and teicoplanin were determined. Molecular typing of VRE isolates was carried out by using pulsed field gel electrophoresis (PFGE) and the resistance genotype was determined by polymerase chain reaction (PCR).* ***Results:*** *Over the six years of our retrospective study; Total enterococcal infection rate represented (13.97%) of which, VRE accounted for 8% of total enterococcal isolates from PICU, and cardiology unit of MUCH. All strains were identified to species level and were found to consist of E. faecalis 30(60%), E. faecium 10 (20%), E. avium 3 (6%), E. hirae 3(6%), E. casseliflavus 2(4%) and E. gallinarum 2(4%) species. According to the susceptibility data obtained, 4 (8%) out of 50 isolates were found to be VRE (MICs > 32 μg/ml). The vanA, vanB gene fragments of E. faecalis, E. faecium were amplified from isolates and were detected. PFGE patterns of the VRE isolates revealed homogenous patterns with dominant clone (A), suggesting the possibility of horizontal transmission among patients. According to local MUCH protocols, monitored doses of various antibiotic combinations as ampicillin/sulbactam and a third generation cephalosporin can be used in serious VRE infections, empirically. In our current study, the use of combinations of teicoplanin (Teichomycin) and amoxicillin had shown great success in treating the isolated VRE strains (2 out of 4 "50%" were sensitive with MICs ≤ 4 μg/ml) or a combination of ampicillin, imipenem, and vancomycin (3 out of 4 "75%" [p<0.05] were sensitive with MICs ≤ 4 μg/ml) .* ***Discussion and conclusion:*** *This study shows an emergence of VRE along with increased rate of multidrug-resistant enterococci in infective endocarditis patients at MUCH. The empirical use or, rather misuse, of antibiotics to treat or to combat the consequences of infective endocarditis in children poses a serious threat rather than benefit the patient and community by increasing the chances of developing new resistant and possibly transmissible genes among enterococci and related species. Although, VRE antibiotic susceptibilities being done for each infection should help guide the selection of treatment protocols, yet surveillance of antimicrobial susceptibilities should be done regularly and the risk factors should be determined and more importantly set up a tight plan of VRE infection prevention and early control, to be followed thoroughly, audited and updated regularly, at the Cardiology Unit and PICU of MUCH.*

INTRODUCTION

Infective endocarditis is a serious disease with an incidence of 30 to 100 episodes per million patient per years.[(1-3)] Mortality rates are usually high and more than third of patients will die within the first year of diagnosis.[(4,5)] Since the first analysis of 209 cases by Sir William Osler in 1885,[(6)] the epidemiological pattern of infective endocarditis has changed [(7,8)] and

prevention strategies have not lowered the incidence of this life-threatening disease.[1,8,9] Mortality has been affected by modifications in antimicrobial therapy. In the years after the introduction of Sulphonamides and Penicillins, synergistic antibiotic combinations and new antibiotics have been tested to optimise treatment. In many in-vitro studies, combinations of antibiotics have shown synergistic activities against the pathogens that commonly cause infective endocarditis.[10]

Enterococci have evolved over the past century from being an intestinal commensal organism of little clinical significance to becoming the second most common nosocomial pathogen associated with significant morbidity and mortality. [11,12] In recent years, there has been a rapid increase in the incidence of infection and colonization of patients with vancomycin-resistant enterococci (VRE). The resistance may be intrinsic or acquired via gene transfer.[11] Widespread use of vancomycin and extended-spectrum cephalosporins in hospitals likely contributed to the emergence and dramatic increase of VRE over the past 20 years. [12, 13] The prevalence of VRE has dramatically increased worldwide.[12, 13] The National Nosocomial Infection Surveillance (NNIS) system in the USA has revealed a significant increase in the percentage of invasive nosocomial *Enterococcus* strains displaying high-level vancomycin resistance.[14]

Although more than one dozen species of enterococci have been identified, *Enterococcus faecalis* was the most common species associated with nosocomial infections, followed by *Enterococcus faecium*, and both species are responsible for about 95% of infections caused by enterococci.[14] VRE, especially *E. faecalis* and *E. faecium*, are prevalent in the hospitalized patients. Other *Enterococcus* species, *E. gallinarum, E. casseliflavus, E durans, E. avium,* and *E. hirae*, are isolated much less frequently and account for less than 5% of clinical isolates.[11,14] Infections caused by VRE were found to be associated with adverse outcome such as extended length of hospital stay, increased cost and increased mortality.[12,14]

The glycopeptide vancomycin is the first choice alternative to penicillin-aminoglycoside combination for treatment of systemic enterococcal infections. Six different types of vancomycin resistance genes are shown by enterococcus: Van-A, Van-B, Van-C, Van-D, Van-E and Van-F. Of these, only Van-A, Van-B and Van-C have been seen in general clinical practice, so far. The significance is that Van-A VRE is resistant to both vancomycin and teicoplanin, Van-B VRE is resistant to vancomycin but sensitive to teicoplanin, and Van-C is only partly resistant to vancomycin, and sensitive to teicoplanin.[15]

In the US, linezolid is commonly used to treat serious cases of VRE, as teicoplanin is not available.[15] Vancomycin resistance is most commonly found in *E. faecium* and is encoded by the *vanA* gene cluster carried on the mobile genetic element Tn1546.[16] Transfer of resistance can occur via conjugative plasmids. Enterococci, as reservoirs of antibiotic resistance genes, tend to transfer their resistance genes to the other bacteria, including methicillin-resistant *Staphylococcus aureus*.[17]

Dissemination of vancomycin resistance can occur through both clonal expansion of VRE and horizontal transfer of *van* genes to other bacteria. Currently, multiple clones of vancomycin resistance genes (clone A, clone B1, and clone B2) and their horizontal transmission are commonly encountered. VRE infection among children, unlike neonates, is possible because of their external contact with health care personnel or through contaminated environments. Asymptomatic patients with VRE colonization can serve as continuing reservoirs because healthcare workers may not adequately wash their hands between contacts with different patients.[16,17]

Monitoring the antibiotic resistance of enterococci isolated from clinical specimens is a useful tool to get information about the prevalence of VRE and will be essential for controlling the spread of bacterial resistance. Despite the increasing reports of VRE in different countries, there is a distinct lack of data regarding the molecular characterization of VRE isolates, originating from the Middle East including Egypt. The aim of this study was to investigate the occurrence, species prevalence, antibacterial resistance and phenotypic and genotypic characterization of VRE isolated from blood samples of infective endocarditis patients in MUCH.

MATERIALS AND METHODS

Study design and data collection

This study was carried out, retrospectively, over 6 years from July 2006 to July 2012. It included 358 blood specimens, collected from 350 in-patients and out-patients at MUCH cardiology unit and PICU (The extra 8 samples were duplicates). We used trypticase soy agar supplemented with 5% sheep blood (BBL, Cockeysville, MD, USA) to isolate enterococci

from blood. Presumptive identification of enterococci was based on their growth characteristics on blood agar, Gram staining, the catalase reaction, ability to grow in 6.5% NaCl broth, bile esculin hydrolysis and biochemical tests, to confirm and detect enterococci to species level, using API Strep (bioMe´rieux, Marcy l'Etoile, France). After the identity of the isolates was confirmed, they were stored in trypticase soy broth containing 16% glycerol at −70°C in freezer vials pending for further analysis. Strains viability was sustained by periodical defrosting, re-culturing, re-testing and re-storing on new media.[18]

Antimicrobial susceptibility testing

The susceptibilities of all isolates to different antimicrobial agents were tested by the disc-agar diffusion method as standardized by the Clinical Laboratory Standards Institution (CLSI). The following antimicrobial discs and concentrations were used: ampicillin (10 µg), vancomycin (30 µg), teicoplanin (30 µg), erythromycin (15 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), amikacin (200 µg), gentamicin (10 µg), kanamycin (200 µg), streptomycin (300 µg), tetracycline (30 µg) and linezolid (30 µg) (Becton Dickinson Microbiology Systems, BBL, Cockeysville, MD, USA). The results were recorded after 24 h of incubation at 35°C and after 48 h for streptomycin. Quality control strains of *E. faecalis* (ATCC 51299) were used to ensure the potency of each antimicrobial agent tested.[18]

Susceptibility interpretations followed the guidelines proposed by the CLSI. Minimum inhibitory concentrations (MICs) for vancomycin and teicoplanin were determined using E-test strips (AB Biodisk, Solna, Sweden) according to the manufacturer recommendations. MICs for gentamicin, kanamycin, streptomycin, amikacin and linezolid were determined by the agar dilution method. For this study, the resistant isolates were submitted to E-test according to the manufacturer recommendations to determine their minimal inhibitory concentration (MIC) to vancomycin and teicoplanin. Vancomycin and teicoplanin resistance were defined as any enterococcal isolate with an MIC to vancomycin and teicoplanin of at least 32 µg/ml. Vancomycin resistance was confirmed by hybridization to specific gene probes. The specific vancomycin-resistant genotype *(vanA, vanB* or *vanC)* was determined with polymerase chain reaction (PCR) analysis by using specific primers selected from published gene sequences.[19]

The patients with VRE endocarditis were followed up after changing the local antibiotic policy used empirically to treat VRE infections, from (a combination of ampicillin/sulbactam and a third generation cephalosporin) to our recommended (combination of teicoplanin "Teichomycin" and amoxicillin or a combination of ampicillin, imipenem, and vancomycin), based on sensitivity patterns obtained during the study, and results were compared.

DNA isolation:

Isolates of VRE were grown overnight at 37°C on bile aesculin agar (Difco Laboratories). The colonies were cultured in 30 ml of brain heart infusion broth (Difco Laboratories) and were harvested during logarithmic growth (optical density at 590 nm of 0.8). DNA was prepared by using a modification of the initial steps of the method of Crosa and Falkow [20] to recover both chromosomes and large plasmids. The bacterial pellet was suspended in 1.5 ml of 25% sucrose-0.05 M Tris-0.001 M ethylene-diamine-tetra-acetic acid (EDTA; pH 8); 0.2 ml of a freshly prepared 40 mg/ml solution of egg white lysozyme (Boehringer Mannheim, Mannheim, Germany) in 0.25 M Tris (pH 8) was added. After incubation for 30 min at 37°C, the bacteria were lysed by adding 0.1 ml of 0.25 M EDTA (pH 8) and 0.1 ml of 20% sodium dodecyl sulphate (SDS). NaCl was added to 1 M; this was followed by incubation on ice for 30 min, centrifugation at 30,000 X*g* at 4°C for 30 min, and incubation of the decanted clear lysate with 5 ml of a 5 mg/ml solution of bovine pancreatic RNase (Boehringer Mannheim) for 30 min at 37°C. DNA concentrations were estimated by electrophoresis on 1% agarose gels (Hispanagar; Sphaero Q, Leiden, The Netherlands) containing ethidium bromide in the presence of known quantities of lambda DNA as references.

Detection of vancomycin-resistance determinants:

Genes encoding the vancomycin resistance determinants, *vanA vanB,* and *vanC*, were investigated by PCR using specific primers, as described by Dutka-Malen *et al*.[19] [Table1]. For the detection of Van genotypes, the VRE and intermediate resistant (IR) strains were investigated for their vancomycin-resistant genotypes by PCR. Amplification was performed using a kit from Gibco-BRL. The amplification mixture consisted of 45 µl Supermix (22 mM Tris/HCl, pH 8.4; 55 mM KCl; 1.65 mM $MgCl_2$; 220 µM each dNTP; 22 U recombinant Taq DNA polymerase/ml; 3 µl bacterial DNA and 2 µl primer solution in a

total reaction volume of 50 µl. A Perkin Elmer 9600 thermocycler was programmed for 32 cycles with the following parameters: denaturation at 94°C for 3 min, annealing at 60°C for 45 s, extension at 72°C for 1 min and final extension at 72°C for 2 min. Amplicons were analysed by electrophoresis on 1% agarose gels (Hispanagar; Sphaero Q) containing ethidium bromide in TAE buffer for 2 h at 70 V in the presence of a 100-bp DNA ladder (Gibco/BRL Life Technologies, Breda, The Netherlands). *E. faecalis* strain E206 (*vanA*) and *E. faecium* E2781 (*vanB*) were used as controls in the PCR experiments.

Table 1: Nucleotide sequences of PCR primers used for the detection of vancomycin-resistant enterococci:

Primer pair	Primer sequences(5'-3'	Product size (BP)	References
A1	5'-GGGAAAACGACAATTGC-3'	732	Dutka-Malen et al.[19]
A2	5'-GTACAATGCGGCCGTTA-3'		
B1	5'-ATGGGAAGCCGATAGTC-3'	635	Dutka-Malen et al.[19]
B2	5'-GATTTCGTTCCTCGACC-3'		
C1	5'-GGTATCAAGGAAACCTC-3'	822	Dutka-Malen et al.[19]
C2	5'-CTTCCGCCATCATAGCT-3'		
D1	5'-CTCCTACGATTCTCTTG-3'	439	Dutka-Malen et al.[19]
D2	5'-CGAGCAAGACCTTTAAG-3'		

Primers D1 and D2 are specific for the *van C2 gene*

Restriction fragment analysis by pulsed field gel electrophoresis:

Chromosomal DNA was obtained as described by Kaufmann.[21] Purified DNA was digested with the restriction enzyme *SmaI* (GIBCO BRL, New York, USA) and separated by pulsed field gel electrophoresis (PFGE) in 1% agarose gels, using a CHEF-DRII system (Bio-Rad Laboratories, Richmond, CA, USA). The pulse time was increased from 5 to 35s, over 22 h, at 5.9 V/cm at a temperature of 11 ºC and an angle of 120 ºC. Lambda ladder of 48.5 kb concatamers (New England BioLabs, Beverly, MA, USA) was used as the molecular weight marker. Gels were stained with ethidium bromide and photographed under UV light. Analysis of DNA restriction profiles was performed by visual inspection according to Tenover *et al*.[22]

Statistical analysis:

Quantitative data were expressed as mean ± standard deviation. Nominal data were analyzed using chi-square test, and *P* values < 0.05 were considered statistically significant. Data were tabulated and analyzed using the SPSS 11 statistical package (SPSS Inc., Chicago, IL) .

RESULTS

Enterococcal isolates:

The distribution of species is shown in [Table2]. Over 6 years from July 2006 to July 2012, A total of 50 enterococcal isolates were isolated from blood cultures, representing a total enterococcal infection rate of (13.97%). Six different species were identified, of which *E. faecalis* was the most prevalent. Out of 50 enterococcal isolates, 30 (60%) were identified as *E. faecalis*, 10 (20%) as *E. faecium*, 3 (6%) as *E. avium*, 3 (6%) as *E. hirae* and 2 (4%) as *E. casseliflavus* and *E. gallinarum,* each. The mean age of the study participants was 7 years and 6 months; 34 of them (68%) were males. Forty five Enterococcal isolates (90%) were recovered from cardiology unit and 5 (10%) from PICU.

Table 2: Distribution of enterococcus spp. isolated from blood cultures:

Total No of samples	No.of isolated enterococci	No (%) of enterococcus species recovered						No of specimens with VRE
		E. faecalis	*E. faecium*	*E. avium*	*E. hirae*	*E. casseliflavus*	*E. gallinarum*	
358	50 (13.97%)	30 (60%)	10 (20%)	3 (6%)	3(6%)	2 (4%)	2 (4%)	4 (8%)

VRE: Vancomycin Resistant Enterococci

Resistance rate for clinical enterococci isolates:

The distribution of antimicrobial resistance patterns of isolated enterococci is summarized in [Table 3]. The results show that the majority of isolates were resistant to tetracycline (68%), erythromycin (64%), ciprofloxacin (48%) and chloramphenicol (32%). High-level resistance to gentamicin (MIC >500 µg/ml), kanamycin (MIC > 1000 µg/ml), amikacin (MIC > 512 µg/ml) and streptomycin (MIC > 2000 µg/ml) was detected in18, 18, 24 and16 % of the isolates, respectively. In addition, 13 (26%) of the isolates were resistant to ampicillin (MIC > 16 µg/ml). The isolates were tested for their susceptibility to linezolid, a new oxazolidinone antibacterial. They were all susceptible to linezolid (MIC range: 0.5-4 µg/ml) except one isolate of E. faecium.

Table 3: Resistance rate for clinical enterococci isolates:

Antibiotics	**Resistant isolates**				**Total N (50)**
	MIC breakpoint(µg/ml)	***E. faecalis* N(30)**	***E. faecium* N(10)**	**Other species* N(10)**	
Ampicillin	>16	5(16.6%)	7(70%)	1(10%)	13 (26%)
Vancomycin	>32	1(3.3%)	3(30%)	-	4 (8%)
Teicoplanin	>32	1(3.3%)	2(20%)	-	3 (6%)
Erythromycin	ND	18(60%)	8(80%)	6(60%)	32 (64%)
Ciprofloxacin	ND	15(50%)	5(50%)	4(40%)	24 (48%)
Chloramphenicol	ND	10(33.3%)	3(30%)	3(30%)	16 (32%)
Amikacin	>512	7(23.3%)	4(40%)	1(10%)	12 (24%)
Gentamycin	>500	6(20%)	2(20%)	1(10%)	9 (18%)
Kanamycin	>1000	6(20%)	2(20%)	1(10%)	9 (18%)
Streptomycin	>2000	7(23.3%)	1(10%)	-	8 (16%)
Tetracycline	ND	18(60%)	8(80%)	8(8%)	34 (68%)
Linezolid	>4	-	1(10%)		1 (2%)

MIC: minimal inhibitory concentration, ND: not determined, (%), percentage of resistant isolates, *Other species; E. avium (no=3), E. hirae (no=3), E. casseliflavus (no=2), E. gallinarum (no=2).

Detection of van genotypes:

Three of the four resistant strains were positive for Van A genotype and a 732-bp PCR product was obtained in all the positive isolates. Van B products (635 bp) were detected in one intermediate resistant isolate as shown in [Table 4]. No Van C products were detected in any of the isolates.

MIC distribution of Van-producing enterococcal isolates (Phenotyes):

Vancomycin and teicoplanin resistance were detected in all 4 (8%) VRE isolates of which, three strains were, *E. faecium* (2 strains were VanA phenotype as they were resistant to vancomycin [MIC ≥32 µg/ml] and intermediate resistant to teicoplanin [MIC ≥ 16 µg/ml], and one strain was Van B phenotype as they had intermediate resistance to vancomycin [MIC ≥ 8-256 µg/ml], and was sensitive to teicoplanin [MIC ≤4 µg/ml] and one *E. faecalis* (Van A phenotype which was resistant to vancomycin [MIC ≥ 32 µg/ml] and intermediate resistant to teicoplanin [MIC ≥ 16 µg/ml]) [Table 4].

Table 4: MIC distribution of Van-producing enterococcus isolates (total No=4)

Species No. (%)	**Vancomycin resistance**		**MIC (µg/ml)**	
	Phenotype	**Genotype**	**Vancomycin**	**Teicoplanin**
E. faecium: 2 (50%)	Van A	Van A	≥32 (R)	≥16 (I-R)
E. faecium: 1 (25%)	Van B	Van B	≥8-256 (I-R)	≤4 (S)
E. faecalis: 1 (25%)	Van A	Van A	≥32 (R)	≥16 (I-R)

-MIC: minimal inhibitory concentration, R: resistant, I-R: intermediate resistant, S: sensitive

-All of the vancomycin-resistant isolates expressed low MIC values for linezolid (MIC ≤ 2 µg/ml).

Resistance rate with antibiotic combinations of isolated VRE by species:

The use of combinations of teicoplanin (teichomycin) and amoxicillin had shown great success in treating the isolated VRE strains (2 out of 4 "50%" were sensitive with MICs ≤ 4 μg/ml) or a combination of ampicillin, imipenem, and vancomycin (3 out of 4 "75%" were sensitive with MICs ≤ 4 μg/ml *P<0.05*) [Table 5].

Table 5: Resistance rate with antibiotic combinations of isolated VRE by species.

Total No of specimens with VRE	No (%) of isolated VRE by species					
	E. faecalis	***E. faecium***	***E. avium***	***E. hirae***	***E. casseliflavus***	***E. gallinarum***
4 out of 50 isolated enterococci (8%)	1(25%)	3 (75%)	0	0	0	0
Vancomycin sensitivity rate after applying new antibiotic combination versus VRE species						
*Teicoplanin + Amoxicillin	1 (25%)	1 (25%)	0	0	0	0
*Ampicillin+ Imipenem+ Vancomycin	1 (25%)	2 (50%)	0	0	0	0

**MIC ≤ 4 μg/ml.*

PFGE of vancomycin-resistant isolates:

The 4 vancomycin-resistant isolates were typed by PFGE after SmaI digestion of their genomic DNA. The genetic profile of four isolates of VRE was examined and we found that two isolates showed 100% identical profile "clone A" match. The other Two isolates proved to be highly correlated to the clone A (90% match) and were; according to molecular size reference, considered to belong to this major clonal group 'A' with possible genetic transformation. All isolates were obtained from different patients who had been admitted to PICU and cardiology unit at close intervals, indicating the possibility of cross transmission among those patients and also, alerting for the urgent need to set up a tight plan of VRE infection early control, trace possible sources of infection and prevent any eminent outbreaks at the cardiology unit and PICU of MUCH.

DISCUSSION

During the period of study and between, July 2006 till July 2012, we examined 358 blood samples from 350 young patients for the presence of VRE at MUCH (The extra 8 samples were duplicates). The vast majority of the isolates in this study were either *E. faecalis* which caused 60% of infection or *E. faecium* which was responsible for 20% of infection, while *E. avium*, *E. hirae, E. casseliflavus* and *E. gallinarium* accounted for only 20% of the isolates [Table 2], which was comparable to the distribution of enterococcal species in other studies.[23,24]

This species distribution is similar to that reported from different parts of the world including the study conducted in north India by, Mohanty et al.[25] But it is in disagreement with reports from some other countries (Taiwan), where *E. faecium* was predominant over *E. faecalis*.[26] The prevalence of *E. faecium* in this study was 20% higher than the prevalence reflected in a similar study conducted in Saudi Arabia by Osoba et al.[27]

Enterococci are intrinsically resistant to several antimicrobials and can develop resistance to many others, which complicates treatment of their infections.[14] Of the 50 isolates, 68, 48 and 32% were resistant to tetracycline, ciprofloxacin and chloramphenicol, respectively [Table 3], which are similar to the levels reported for these antibacterials among the enterococci isolated in Jordan (2008) as reported, by Mahafzah et al [28] and also, in a previous study by Khan et al (2008) in Saudi Arabia.[29] Frequency of resistance to the commonly used antimicrobials was slightly higher among isolates of this study than that reported from other countries like the study of Udo et al (2003) in Kuwait [14] and that of Leven et al (1999).[30] In this current study, 64% of isolates were resistant to erythromycin [Table 3], which is lower than what was reported from other countries such as India (85%) and Lebanon (59%).[23,24] These results indicate diverse geographical distribution of erythromycin-resistant enterococci. Antimicrobial resistance has been consistently

reported to be more common in *E. faecium* as compared to *E. faecalis* [14,28,29], which also concurs with our results [Table 3].

Enterococci with VRE are being reported from different parts of the world with increasing frequency, although the epidemiology of these microorganisms varies widely in different geographical areas.[23,31] The percentage of reported hospital enterococcal infections resistant to vancomycin increased from 0.3% in 1989 to 11% in 1996. [32] In this study, VRE were isolated from 4 patients and accounted for 8% of total enterococcal isolates and were all identified as *E. faecalis* and *E. faecium* [Table 3]. Medical records of these 4 patients were reviewed and their clinical features were determined. The patients were immunocompromized and their age varied from 3 to 12 years. All the patients except one were in-patients when VRE was detected in their specimens. These results are in agreement with those of Udo *et al*. [14] who detected VRE in 11 out of 415 isolates (2.6%) at Kuwait hospitals. However, our results don't agree with those of Gambarotto *et al*. [33] who reported that the incidence of VRE in hospitalized patients was 26/70 (37%) and 20/169 (11.8%) in non-hospitalised controls. Also, Leven *et al*. [30] reported the incidence of VRE in 586/1260 (46.5%) patients in a University hospital in Belgium, and both results introduced by Gambarotto *et al*. [33] and Leven *et al*. [30] are astonishingly high compared to our current results.

All enterococcal isolates included in this study were susceptible to linezolid except one (*E. faecium*) [Table 3]. Also, 29/30 (96.7%) of the *E. faecalis* in this study were vancomycin susceptible [Table 3]. The low prevalence of vancomycin resistance among the isolates in this study indicated that vancomycin retains its therapeutic efficacy against the majority of enterococci isolated from patients in MUCH. The resistance rate to ampicillin was found to be 26% in enterococcal isolates [Table 3], however, resistance rate to ampicillin reported by Mathur *et al.* [34] in India was as high as (66%), which is much higher rate compared to our results.

Since ampicillin is the drug of choice in the treatment of enterococcal infections, the relatively high resistance of isolates in this study to ampicillin is of great concern, especially in the case of endocarditis treatment, particularly among children, which rings the bell to reduce the abuse of such, rather safe and cheap antibiotic in a step to stop the progression of that considerably high resistance rate. Our results, also revealed that the isolates were expressing high-level resistance to amikacin, gentamicin, kanamycin and streptomycin (24, 18, 18, and 16%, respectively) [Table 3]. The detection of high-level gentamicin resistance in 20% of *E. faecalis* and *E. faecium* isolates [Table 3] is a cause for concern, as it may signify the beginning of another major resistance problem and that another serious action plan is also, urgently required to prevent that uprising problem.

According to local MUCH protocols, monitored doses of various antibiotic combinations as ampicillin/sulbactam and a third generation cephalosporin can be used empirically to treat VRE infections. The fact that the uncalculated misuse of those antibiotics, especially for long time and with high doses, increases, greatly the chance of developing new resistant strains; In our current study, based on the sensitivity pattern, using combinations of teicoplanin (teichomycin) and amoxicillin had shown great success in treating the isolated VRE strains (2 out of 4 "50%" were sensitive with MICs $\leq$ 4 μg/ml) or a combination of ampicillin, imipenem, and vancomycin (3 out of 4 "75%" were sensitive with MICs $\leq$ 4 μg/ml [$p<0.05$]) [Table 5]. Even though 98% of all isolated enterococci (VRE and non VRE) had shown 100% sensitivity to linzolid, there is still a cap enforced by the FDA on using such antibiotic because of its adverse side effects; which limited its use only to fatal cases of infective endocarditis, also and because of the lack of availability of such antibiotic in MUCH, that makes such combinations [Table 5] proposed in our current study a life saving therapeutic alternative that can also be used in serious and fulminant cases of infective endocarditis caused by mlti-resistant VRE but with less side effects especially when monitored while used. Such recommendations were sent to the heads of Cardiology unit and PICU at MUCH and were tracked to check whether the new antibiotic policy has been put into place and to ensure the stop of the misuse of other antibiotics, especially by junior doctors.

Vancomycin-resistant phenotypes in enterococci have been classified as VanA, VanB, VanC, VanD and VanE, based on levels of resistance, cross-resistance to teicoplanin and inducible or constitutive nature of the resistance.[23,27] In enterococci, two principal phenotypes of acquired vancomycin resistance have been described, VanA and VanB. The VanA determinant is carried on transposon Tn1546 or close relatives that are transferable in conjugation experiments.[6,32,35]

In the current study, we have investigated the prevalence of genes encoding vancomycin resistance by PCR (genotype) in isolated VRE and correlated it to the phenotype (based on Vancomycin and Teicoplanin MIC sensitivity patterns [Table 4]), and we found that, VanA genotype was present in 3 out of 4 (75%) of the isolated VRE which were compatible with VanA phenotype and were resistant to vancomycin (MIC ≥ 32 µg/ml) and intermediate resistant to teicoplanin (MIC ≥ 16 µg/ml), whereas VanB genotype was found only in 1 out of 4 (25%) of the isolates and was compatible with VanB phenotype which showed only intermediate levels of vancomycin resistance (MIC ≥8-256 µg/ml) and was sensitive to teicoplanin (MIC ≤ 4 µg/ml) [Table 4].

By further analysis of PCR results of isolated van producing VRE (genotype) by species; We found that one *E. faecalis* (25%) and two *E. faecium* (50%) isolates expressed vancomycin-resistance patterns compatible with its VanA phenotype [Table 4], while one *E. faecium* (25%) isolate expressed vancomycin-resistance patterns compatible with its VanB phenotypes [Table 4]. By this, our study agrees with other published variable results concerning the detection rates of VanA and VanB, where, in Kuwaiti hospitals, all VRE strains carried the VanA genotype and non VanB product was detected in any of the isolates [(14)], meanwhile results submitted by Nelson *et al*. [(35)] reported that the majority of studied VRE isolates (97%) were VanB positive and the remaining isolates were VanA genotype.

Currently, multiple clones of vancomycin resistance genes (clone A, clone B1, and clone B2) and their horizontal transmission are commonly encountered. The genetic profile of the four VRE strains isolated in this study, was examined and we found that two isolates showed 100% identical profile matching with "clone A". The other Two isolates proved to be highly related to the clone A (90% match) and were; according to molecular size reference, considered to belong to this major clonal group 'A' with possible genetic transformation. The identification of a distinct major clonal group in VRE draws attention to the increasing need for control measures to avoid horizontal transmission, since the enterococci currently represent a serious problem in health institutions, especially because of the possibility of spread from healthy carriers and the lack of effective treatment options; By this our study concurs with Arthur *et al* [(16)] and Chang *et al* [(17)], who agreed to the fact that, dissemination of vancomycin resistance can occur through both clonal expansion of VRE and horizontal transfer of *van* genes to other bacteria and also from healthcare workers or contaminated environment.

Although the prevalence of vancomycin resistance was low among the studied enterococcal isolates, their presence together with high-level aminoglycoside resistance, calls for regular surveillance studies, infection control and monitoring of antibiotic sensitivity among hospital-isolated strains. Presence of *vanA, vanB* gene cluster in our isolates can provide transfer of vancomycin resistance via conjugative plasmids, not only to enterococci species, but also to other bacteria such as *S. aureus.* So, we should expect an increase in the number of VRE in the future, if not contained as soon as possible.

The appearance of these highly resistant strains prompted us to conduct a series of studies to assess the extent of colonization and infection with VRE in our patient population, to define risk factors for acquisition and to evaluate the effect of interventions on rates of colonization and infection.

CONCLUSION

This study shows an emergence of VRE along with increased rate of multidrug-resistant enterococci in infective endocarditis patients at MUCH. The empirical use or, rather misuse, of antibiotics to treat or to combat the consequences of infective endocarditis in children poses a serious threat rather than benefit the patient and community by increasing the chances of developing new resistant and possibly transmissible genes. Nevertheless and although, VRE antibiotic susceptibilities being done for each infection should help guide the selection of treatment protocols, yet surveillance of antimicrobial susceptibilities should be done regularly and the risk factors should be determined and more importantly set up a tight plan of VRE infection prevention and early control, to be followed thoroughly, audited and updated regularly, at the Cardiology Unit and PICU of MUCH.

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